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COMMENTARY

Innovative Treatment Programs Against Cancer

I. RAS ONCOPROTEIN AS A MOLECULAR TARGET

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ABSTRACT. Modulation of Ras function may provide a novel means by which cancer cells with oncogenic mutations can be sensitized to chemotherapeutic or radiotherapeutic regimens. Moreover, cancer cells without *ras* oncogene mutations can also be eliminated by compounds that interfere with the mevalonate pathway, which is more fundamental to mitogenesis because it allows the synthesis of sterol and nonsterol lipids and without which many Ras-related proteins and nuclear lamins would not be prenylated and functional. BIOCHEM PHARMACOL **56**;11:1411–1426, 1998. © 1998 Elsevier Science Inc.

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The more than 50 low molecular weight proteins categorized into the Ras-related superfamily serve many functions and have representatives present in all eukaryotic cells. These proteins exert control over cellular signalling pathways affecting cytoskeletal organization, intracellular vesicular trafficking, macromolecular biosynthesis, cell cycle entry, and mitogenesis. Ras-related proteins can be conceptualized as regulatory molecular on/off switches that are turned on (activated) by the exchange of bound GDP for GTP and are turned off (inactivated) following hydrolytic dephosphorylation and reversion to a GDP-bound state, either as a result of interaction with their membrane-associated GAP protein-containing regulatory complex or with their downstream target kinases. These regulatory switches operate near the proximal ends of biochemical signal transduction cascades and serve to integrate upstream stimulatory signals. The small GTP-binding proteins are divided into subfamilies, based on their sequence and biological function.

Ras is the prototype small GTP-binding protein, with a 21-kDa molecular mass ($p21^{ras}$), which is post-translationally modified (prenylated) with a hydrophobic lipid moiety, allowing it to be membrane-anchored and functional in the transduction of signals initiated by engagement of membrane receptors with extracellular ligands. Ras regulates differentiation, adhesion molecule expression, and cytoskeletal actin stress fiber organization in particular cell types; however, most significantly, activated Ras is essential for entry into the mitotic cell cycle and sufficient for the maintenance of proliferation. Microinjection experiments demonstrated that Ras activity is required for the $G_0 \rightarrow G_1$ transition of quiescent cells into the active cell cycle as well as late $G_1 \rightarrow S$ phase entry [1]. Selective inhibition experiments showed different family members to be required for G_0/G_1 and G_2/M phase transitions [2].

ACTIVATION OF Ras PROTEIN

Growth factors that promote mitosis stimulate the guanyl nucleotide exchange factor that activates Ras through swapping bound GDP for GTP [3–6]. Growth factors and cytokines as fundamental and general in their stimulatory effects as insulin, EGF†, and PDGF exert their actions via activation of Ras proteins [6–8]. Activation of the Ras pathway is also essential for mitotic responses to engagement of the T-cell antigen receptor complex on T-cells or receptor-binding of lysophosphatidic acid and thrombin on fibroblasts [9, 10]. Receptors that transduce stimulatory signals, including those that initiate cell division, have inherent or associated PTK functions that are activated following ligand binding. Activation of PTK is an upstream inducer of Ras activation through phosphorylation of guanyl nucleotide exchange factor subunits promoting GDP/GTP exchange [11]. This exchange factor has been shown to be constitutively activated in NIH 3T3 cells transformed by Src-family or Erb-family PTK, suggesting that tyrosine phosphorylation of the guanyl nucleotide exchange factor

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†*Abbreviations:* AOM, azoxymethane; BZA, benzodiazepine; CDKs, cyclin-dependent kinases; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; Fmev, 6-fluoromevalonatge; FPP, farnesyl diphosphate; FTase, farnesyltransferase; GDI, GDP-dissociation inhibitory; GGOH, geranylgeraniol; GGTase, geranylgeranyltransferase; IR, ionizing radiation, MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK, mitogen-activated/extracellular response kinase kinase; NAC, tyl-cysteine; PDGF, platelet-derived growth factor; PH, pleckstrin-homology; PI3, kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLA_2, cytoplasmic phospholipase, A_2; PTK, protein tyrosine kinase; VEGF, vascular endothelial growth factor; and TGF, transforming growth factor.

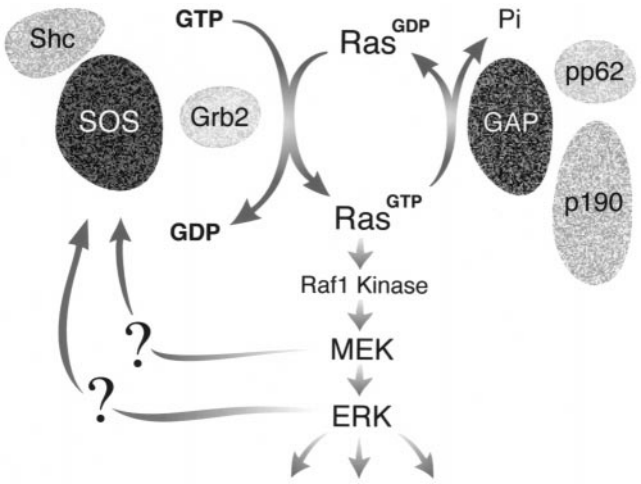

FIG. 1. Ras signalling pathway. See text for details.

may be a meaningful target to prevent malignant transformation [6]. In contrast, transformation by the downstream Mos or Raf oncoproteins does not affect the function of the guanyl nucleotide exchange factor. The use of PTK-specific inhibitors such as emodin [12] or genistein [6] prevents nucleotide exchange, activation of Ras, and cell division.

The term "Ras" actually encompasses a group of proteins that consists of H-Ras, K-Ras, N-Ras, and R-Ras, which have many variants, and their putative specialized functions are currently ill-defined. Those components of the Ras pathway that are close to the GDP/GTP exchange-mediated activation of the Ras protein are more clearly defined. Conversely, the upstream or downstream components distant from Ras activation are vaguely defined. Tyrosine phosphorylation of one or more of the subunits collectively termed Shc is the initial step towards Ras activation [11]. The three Shc proteins each contain a protein tyrosine binding (PTB) domain, an SH2 domain, and three tyrosine autophosphorylation sites. The PTB and SH2 domains facilitate the association of the Shc proteins with the ligand-bound tyrosine phosphorylated receptors and their activated PTK components. The resultant phosphorylation of the Shc proteins allows their interaction with the SH2 domain of the Grb2 adapter protein. SOS is the guanyl nucleotide exchange factor specific for Ras proteins, and the SH3 domains of the Grb2 molecule have an affinity for the proline-rich carboxy-terminal domain of SOS that localizes it to the vicinity of its membrane-associated target Ras substrate (Fig. 1) [3–5]. Artificially targeting SOS for membrane association is sufficient for induction of Ras activation even in the absence of Shc and Grb2. Co-localization of SOS and Ras on the interior of the plasma membrane suffices for the rapid conversion of Ras from its GDP-bound inactive state to its GTP-bound active state.

BIOCHEMICAL AND BIOLOGICAL CONSEQUENCES OF Ras ACTIVATION

The best-defined branch of the downstream signalling pathways initiated by Ras activation is mediated through direct up-regulation of Raf1 serine/threonine kinase activity, which results in reversion of the Ras protein to its GDP-bound inactive state [13, 14] (see Fig. 1). After activation through the GTP-dependent interaction with Ras, Raf1 initiates activation of the MAPKK/MAPK signalling cascade through phosphorylation of MEK1 and -2. The MEKs have both threonine and tyrosine kinase activities and phosphorylate the downstream MAPKs, of the ERK1 and ERK2 family, on a TEY motif, thereby being classifiable as MAPKKs. The phosphorylation and activation of ERK kinase in the vicinity of the plasma membrane allow for and target its translocation past the nuclear membrane. This pathway serves as a means by which activated ERK can functionally interact with both cytosolic substrates and nuclear transcription factors in order to stimulate progression into and through the mitotic cell cycle.

An alternative pathway that is dependent on Ras family activation and contributes to mitogenesis is mediated by activation of Rac serine/threonine kinase [15–17]. This pathway is not as clearly delineated, but it appears to rely on intracellular generation of superoxide ($O_2^{-\cdot}$) radicals rather than activation of the MAPKK/MAPK cascade. The Rac protein has homology with PKC at the C-terminus and contains a PH domain at the N-terminus [17]. Rac is encoded by the *akt* (i.e. *rac*) proto-oncogene, and v-*akt* is thought to induce cancer [17]. That the Akt kinase is controlled by Ras activity was confirmed by dominant-negative mutants of Ras inhibiting Akt activity. Activation of Akt can also be inhibited using wortmannin, a potent and selective inhibitor of PI 3-kinase [17]. Results identify Akt as a novel target of PI 3-kinase and suggest that the Akt PH domain mediates PI 3-kinase interaction and signalling [18].

PI 3-kinase is a membrane-associated heterodimer with a p85 and a p110 subunit [17, 19]. The p85/p110 PI 3-kinase complex has a suppressed catalytic function *in vivo* when compared with free p110, thereby demonstrating that the p85 subunit has a negative regulatory function. Ras can bind to the p85 regulatory and p110 catalytic subunits of PI 3-kinase and up-regulate the enzymatic activity. The phenotype induced by v-*ras* transfection is consistent with activation of PI 3-kinase and is sensitive to the PI 3-kinase inhibitor wortmannin [19].

PI 3-kinase signalling is important in protein synthesis, transformation, cell cycle progression, and mitosis. Activation of the PI 3-kinase complex appears to play a crucial role in transducing cytokine receptor signalling because nearly all of these receptors recruit PI-3 kinase upon ligation [17, 19]. Moreover, the cellular response to growth factors such as insulin, EGF, and PDGF, which activate Ras, can be blocked by wortmannin [17]. The aforementioned findings strongly suggest that PI 3-kinase is a downstream effector of Ras.

Downstream nuclear targets in the Ras pathway include the up-regulated transcription of the *fos* and *jun* genes [20–22]. This is probably mediated through phosphoryla-

tion of the Elk-1/TCF transcription factor by activated ERK1 or -2 kinases. The resultant AP-1 heterodimeric complex participates in cell proliferation and transformation. In addition, Ras contributes to activation of the JNK MAPK, which phosphorylates the transactivation domain of the Jun protein. This phosphorylation enhances AP-1 activity and also amplifies the induction of *jun* transcription through an autoregulatory loop. Through this transduction pathway, AP-1 promotes expression of mitogen-inducible genes that prompt entry into the active mitotic cell cycle.

Growth-factor-initiated progression through the cell cycle is controlled by CDKs. Ras is required for activation of both Cdk2 and Cdk4 until approximately 2 h before the G_1/S phase transition, and Ras is also required for induction of cyclin D1 expression and down-regulation of the CDK inhibitor $p27^{kip1}$, which are necessary events for entry into S phase [23]. Constitutive expression of cyclin D1 circumvents the requirement for Ras signalling in cell proliferation. This indicates that regulation of cyclin D1 expression is a critical target of the Ras signalling cascade.

The *ras* oncogene-encoded p21 protein is implicated in the etiology of a large variety of human tumors. For example, mutational activation of Ras has been found in more than 50% of colon cancer cases and in approximately 90% of pancreatic cancer cases, with the mutation most commonly involving a K-Ras isoform [24, 25]. The Ras oncoprotein typically differs from its normal counterpart protein by having a single amino acid substitution at a critical position that confers constitutive activation by an as yet not fully understood mechanism [26, 27]. The critically sensitive sites in the human Ras protein seem to be Gly12, Gly13, Ala59, and Gln61 [26]. In the AMO-induced rat colon cancer model, the *ras* mutations were always single-point mutations at the second nucleotide of codon 12 [27]. A mutation in K-*ras* always resulted from a G to A conversion, and an H-*ras* mutation was caused by either a G to A conversion or a G to T conversion. Notably, constitutively active Raf, MEK, or ERK kinases can mimic the effect of oncogenic Ras on cell cycle progression, and specific inhibitors of Raf, MEK, or ERK can block the effect of oncogenic Ras. Thus, cellular transformation can result from oncogenic Ras protein activation of the Raf/MEK/ERK pathway [28].

Further confirmation of the importance of mutant Ras activity in uncontrolled cancer cell proliferation was provided by a study [12] showing a natural PTK inhibitor to selectively block growth of v-*ras*-transformed human carcinoma cells. Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) had a half-maximal (IC_{50}) inhibition of cell growth at 4 μg/mL using *ras*-transformed cells, but 100 μg/mL had little effect on the growth of normal human cells. Since the cells transformed with v-*ras* had elevated levels of phosphotyrosine-containing proteins compared with normal counterparts, and emodin treatment caused decreased intracellular tyrosine phosphorylation, the inhibition of Ras-dependent elevation of protein phosphorylation may have been responsible for the Ras-selective growth inhibition, or it may have been due to inhibition of the upstream activators of Ras activity (see above).

Damnacanthal is another anthraquinone compound that inhibits Ras function [29]. It is a chloroform extract from the root of the *Morinda citrifolia* tropical plant. It is able to induce normal morphology and cytoskeletal structure in K-*ras*-transformed cells, whereas damnacanthal has no effect on *src*-transformed cells. Treatment with damnacanthal does not affect the amount or localization of Ras, and its effects are reversible.

Rho proteins are related to Ras and they serve to regulate stress fiber formation, which is deficient in *ras*-transformed cells; it also appears that the $p21^{rho}$ molecule partly serves as a regulatory component for the prevention of polyploidy [30, 31]. Treatment with botulinum C3 coenzyme inactivates Rho and induces polyploidy in a leukemia cell line similar to that seen when actin polymerization is inhibited with cytochalasin B [31]. The presence of Rho proteins, possibly RhoB, was found to be necessary for *ras* transformation, and inhibition of its activity at least partially contributed to suppression of *ras* transformation [30]. The activity of Rho proteins is controlled by prenylation and conversion from a GDP-bound to a GTP-bound state, as is the activity of Ras [30, 32]. Genes that encode two proteins having GDI properties were found in human cells [32]. These proteins prevented activation of Rho by suppressing dissociation from GDP in exchange for GTP. The 27-kDa Rho-GDI isoform is ubiquitously expressed in all tissues, whereas the Ly-GDI isoform is expressed only in hematopoietic tissues and predominantly in B- and T-lymphocytes [32]. Stimulation of T-lymphocytes with phorbol ester leads to phosphorylation of Ly-GDI, suggesting an involvement of Ly-GDI in lymphocyte activation pathways, possibly as a substrate for PKC. Cell-type specific regulators of Ras-like GTP-binding proteins may provide a mechanism for the control of their oncogenic capabilities.

Growth of solid tumors *in vivo* beyond 1–2 mm in diameter is thought to require induction and maintenance of an angiogenic response. Expression of mutant *ras* oncogenes in human cancers is associated with marked VEGF up-regulation [33]. Elevation of VEGF mRNA and secreted functional protein was detected in human and rodent tumor cell lines with mutant K-Ras and H-Ras oncoproteins, respectively. Genetic disruption of the K-*ras* allele in human carcinoma cells was associated with reduced VEGF activity, and pharmacological disruption of mutant H-Ras function in rat carcinoma cells with L-739, 749 (amino-3-mercapto-propylamino-3-methyl-pentyloxy-3-phenylpropionylmethioninesulfone methyl ester; a protein farnesyltransferase inhibitor) caused significant suppression of VEGF production [33]. Dominantly-acting *ras* oncogenes may contribute to the growth of solid tumors *in vivo* indirectly by facilitating tumor angiogenesis, as well as proliferation. Pharmacological targeting of mutant Ras could suppress solid tumor growth *in vivo*, in part by inhibiting tumor-induced angiogenesis.

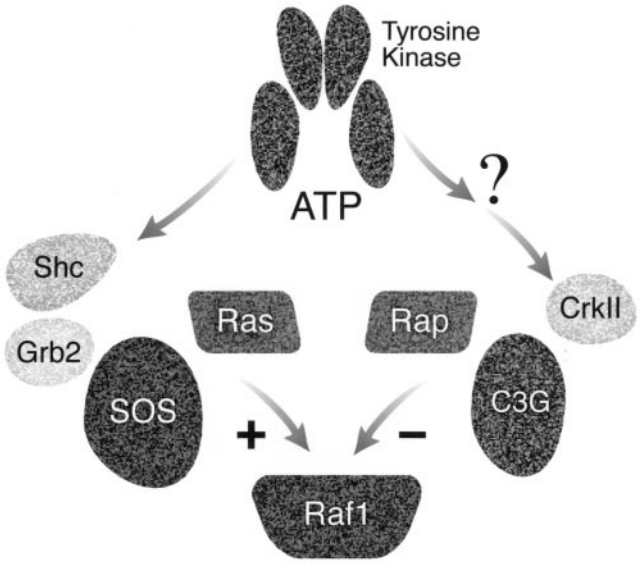

FIG. 2. Regulation of the Ras signalling pathway through antagonistic interactions of Ras and Rap proteins with Raf1.

REGULATION OF Ras ACTIVITY

The Ras pathway is subject to down-regulation by a negative feedback loop regulated by phosphorylation of SOS by a serine/threonine kinase [34–37]. The phosphorylation of SOS apparently causes dissociation of the Shc–Grb2–SOS complex. The nucleotide exchange activity of SOS is lost either due to its phosphorylation or *de facto* because SOS is no longer localized to the membrane. Regardless, GDP-bound Ras does not become re-activated via GTP exchange, and the signalling cascade is quenched. The kinase responsible for phosphorylation of SOS has a number of candidates, including MEK and ERK; however, there is still uncertainty with regard to the identity of the kinase operative *in vivo*. A recent report [37] demonstrated that $p90^{rsk2}$, which is a downstream target of MAPKs such as ERK1 and ERK2, phosphorylates SOS *in vitro* and that the *in vivo* phosphorylation sites on SOS correlate more closely with those of $p90^{rsk2}$ kinase than with those of the MAPK family. Definite identification of this kinase could have a significant impact on cancer therapy in cases dependent on Ras activation, as will be described below.

Supplementing the mechanism of feedback inhibition is another low molecular weight GTP-binding protein called Rap that opposes the Ras activation response (see Fig. 2). Four members of the Rap family are currently identified, namely Rap1A, Rap1B, Rap2A, and Rap2B [38, 39]. In a number of cell types, Rap proteins have been shown to operate by suppressing Ras-induced downstream signalling, with Ras-dependent transformations being reversible by Rap expression. The reversion is due to Rap antagonizing Ras-dependent activation of the MAPKK/MAPK pathway. As a physiologic example, elevation of cAMP levels activates Rap, and this prevents Ras message transduction by blocking activation of the Raf/MEK/ERK cascade. Whereas association of Raf1 with GTP-bound Ras activates Raf1 kinase activity, interaction of Raf1 with GTP-bound Rap inhibits the kinase activity [40].

Rap activation is similar to Ras activation in that its conversion to a GTP-bound state results from interaction with a Rap-specific guanyl nucleotide exchange factor, i.e. C3G [41, 42]. The elevated expression of C3G suppresses the Ras-transformed phenotype in a manner analogous to overexpression of Rap protein. Because Rap functions as a down-regulator of the Ras signalling pathway, a feedback mechanism must exist for rapid inactivation of Rap activity to allow for Ras activation of the Raf/MEK/ERK cascade in non-transformed cells in order to respond to growth factors. By analogy to the negative feedback loop limiting Ras activation through phosphorylation-mediated dissociation of the complex containing SOS and Grb2, a proximal signalling pathway has been proposed recently [43] by which inactivation of Rap is accomplished prior to Ras activation. That is, C3G has been observed to rapidly dissociate from its partner molecule, CrkII, following stimulation with insulin or EGF. These findings on the reciprocal nature of Rap and Ras activities that are controlled by their respective guanyl nucleotide exchange factors, C3G and SOS, depending on whether or not the cell is in active mitosis, have implications for therapeutic modulation of *ras*-transformed cancer cell growth.

There are other upstream, membrane-associated contributors to the Ras signal transduction pathway. In T-lymphoid cells, in particular, Ras activation is partially controlled by activity of the PKC isoforms [9, 44–48]. The Vav protein is a product of the *vav* proto-oncogene that is specifically expressed in cells of hematopoietic origin, and it serves as a substrate for Lck, a Src family kinase, following engagement of the TCR complex [49–51]. Results imply that the activation state of Vav protein modulates the ability of Ras to invoke mitogenesis.

Whereas mitogenic cytokines induce up-regulation of Ras activity, TGF-β1, which is a potent inhibitor of cell growth, decreases the activation state of endogenous Ras, as measured by levels of GTP-bound Ras protein [52]. When cells are released from TGF-induced growth arrest, the MAPK phosphorylation and activity dependent on Ras function revert to a more elevated status. It seems that the growth suppression conferred by exposure to TGF-β1 is mediated by down-modulation of Ras activity.

Some compounds can increase Ras activity through up-regulation of Ras expression. In NIH 3T3 cells, the commonly utilized chemotherapeutic drug dexamethasone triggers Ras expression [53]. Provision of AOM subcutaneously to rats significantly enhances expression of Ras in colon cells [27]. AOM-induced Ras expression could be inhibited by dietary administration of chemopreventive drugs such as D, L-α-difluoromethylomithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, and piroxicam, a non-steroidal anti-inflammatory drug (NSAID). Repeated injections with AOM caused colon carcinomas in a large percentage of the rats, and the carcinoma cells nearly always harbored identifiable mutations in the H-Ras

or K-Ras proteins. The carcinomas induced by Ras mutations were far less numerous in rats treated with DFMO or piroxicam.

PHARMACOLOGICAL MODULATION OF Ras ACTIVITY TO PROMOTE CELL DEATH

NIH 3T3 fibroblasts transformed with constitutively active isoforms of Ras, such as v-H-Ras or EJ-Ras, produce large amounts of the superoxide radical [54]. $O_2^{-\cdot}$ production could be suppressed by dominant-negative mutants of Ras or Rac, or by treatment with a FTase inhibitor that prevents Ras from being prenylated and activated by membrane localization. The $O_2^{-\cdot}$ production could also be suppressed by treatment of the cells with diphenylene iodonium, a flavoprotein inhibitor. The mitotic activity of cells transformed with H-Ras was inhibited by treatment with NAC, an antioxidant radical scavenger. These results imply that reactive oxygen intermediates are involved in the regulation of cell-cycle progression, in particular in *ras*-transformed cells, even when MAPK and JNK/SAPK kinases are not activated. Therefore, the use of antioxidants may be useful in suppressing the *in vivo* proliferation of *ras*-transformed cells.

In addition, survival-promoting protective signals in response to exogenous oxidative agents that modulate cellular redox status, for example, H_2O_2, Hg^{2+}, NO, or hemin, can be prevented by blocking Ras activity [55]. This is achieved either using a dominant-negative $p21^{ras}$ mutant or with a FTase inhibitor, as assessed by NF-κB DNA-binding activity. The NF-κB response to oxidative stress was enhanced when Jurkat T-cells were pretreated with L-buthionine sulfoximine to inhibit GSH synthesis. Ras and MAPK activities are up-regulated in cells by redox modulating agents, such as those previously mentioned. The redox modulators directly promote guanyl nucleotide exchange on Ras. Direct activation of Ras may be a central mechanism whereby a variety of oxidative stress signals can initiate protective signal transduction events affecting gene expression and cell survival. Inhibition of Ras reactivity, therefore, may sensitize malignant cells to treatment with ionizing radiation and some chemotherapeutic drugs, by upstream interference in the NF-κB activation pathway.

DEPLETION OF THE CELLULAR GTP POOL

The most fundamental means by which activation of Ras-family proteins can be suppressed is prevention of the GDP/GTP exchange. Tiazofurin is a specific inhibitor of IMP dehydrogenase and causes depletion of the cellular GTP pools [7]. Because the active form GTP-Ras is functionally inactivated by hydrolyzing bound GTP to form GDP upon interaction with its proximal substrate, a decreased supply of GTP would repress Ras activity. A diminished pool of GTP can also down-regulate *ras* gene expression and trigger cellular differentiation. Inhibition of the GTP salvage pathway using hypoxanthine enhanced the tiazofurin-induced decrease of GTP-Ras.

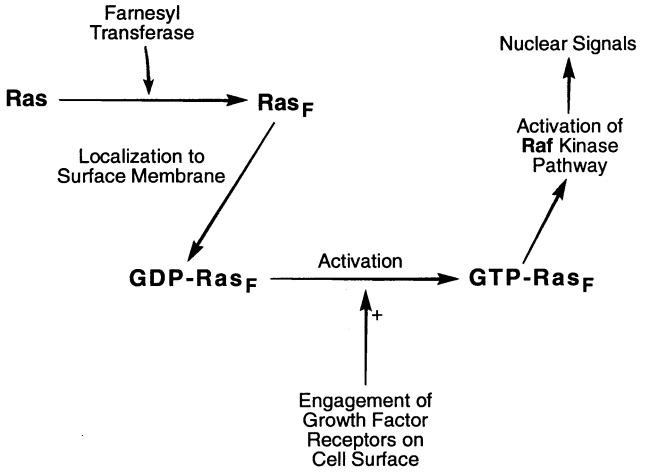

FIG. 3. Schematic depiction of the linkage of Ras proteins to lipid moieties, such as farnesyl, through the actions of farnesyl transferase, that allow membrane localization. When Ras is associated with the internal cell membrane, it can be activated to initiate kinase signalling cascades necessary for cell proliferation. Conversely, activation of Ras can be prevented by inhibiting isoprenylation with molecules such as farnesyl, geranyl, and geranylgeranyl diphosphates.

PRENYLTRANSFERASE-MEDIATED ACTIVATION OF Ras

For acquisition of biological activity, Ras-family proteins must bind to the internal plasma membrane, and prenylation with a lipid moiety is essential to this ability, even with constitutively active oncogenic Ras proteins [56–62] (see Fig. 3). This post-translational modification of Ras is performed by the FTase and GGTase enzymes. Prenylation of Ras-family proteins by FTase can use farnesyl, geranyl, geranylgeranyl, or dimethylallyl diphosphates (PP) as substrates, whereas GGTase appears to be restricted to the two geranyl-type substrates [63]. Prenylation of G-proteins, including Ras and the nuclear lamins A and B, requires an active transferase, such as FTase, and sufficient substrate, such as FPP, for modification of these proteins to give lipophilic anchors that bind to membranes. Without the enzyme and substrate, nuclear lamins and Ras proteins remain in the cytosol in nascent states, and cells do not proliferate [59, 60]. Furthermore, to acquire transforming potential, the precursor of the Ras oncoprotein must undergo prenylation, and a major area of active research is the development of pharmacological approaches for antagonizing oncogenic Ras activity through inhibition of prenylation.

The site of Ras protein prenylation is a cysteine residue four amino acids into the carboxy-terminal CAAX tetrapeptide motif [58, 60–63]. In this C-terminal motif, C represents cysteine, A represents the aliphatic leucine, isoleucine, or valine residues, and X stands in for methionine or serine. Following prenylation, the C-terminal three amino acids are proteolytically cleaved and the prenylated

cysteine is methylated. When prenyl substrate sequence preference was examined using FTase [63]. FTase catalyzed both farnesylation and geranylation of Ras-CVLS and of Ras-CVIM. Geranylgeranylation was observed only when Ras-CVIM was the acceptor substrate. In contrast, Ras-CAIL could not be prenylated. As a consequence of its wider range of prenyl-family substrates and Ras CAAX motif sequences serving as prenylation substrates, inhibitors of FTase activity have been the primary focus of efforts to suppress the function of Ras through prevention of prenylation.

FTase is a zinc metalloenzyme, and the zinc is essential to its catalytic activity [64]. FTase has been termed a "housekeeping enzyme" [30, 65]. It is a heterodimer with a 49-kDa α-subunit and a 46-kDa β-subunit [66]. The endogenous heterodimeric FTase protein is phosphorylated on the α-subunit *in vivo*, and this has a role in negatively regulating its activity. Treatment of cells with the protein phosphatase inhibitor calyculin caused a decrease in FTase activity, and treatment with the serine/threonine-specific protein phosphatase-1 greatly increased FTase activity in cell extracts [66]. The pharmacological inhibitors specific for FTase activity that will be described below generally inhibit growth of *ras*-transformed cells *in vitro* and *in vivo*.

NATURAL INHIBITORS OF FARNESYLTRANSFERASE ACTIVITY

Several natural compounds have been isolated that appear to directly inhibit FTase enzymatic activity. A strain of *Streptomyces* produces manumycin, which suppresses FTase activity [67, 68]. Manumycin inhibits Ras farnesylation and downstream MAPK activity, as shown in a human *ras*-transformed hepatoma cell line [67]. Manumycin, however, decreased neither geranylgeranylation of the Ras-antagonizing Rap protein nor prenylation of other 21- to 26-kDa proteins. *In vitro* studies showed manumycin to significantly inhibit growth of a human Ki-*ras*-transformed fibrosarcoma cell line [68], and it had a lower IC_{50} for growth of human pancreatic cancer cell lines with mutant K-Ras than in those with wild-type Ras [57]. Moreover, at high enough concentrations, manumycin induced apoptosis. Manumycin was tested on the growth in nude mice of human pancreatic cancer cells with a point mutation in the Ki-*ras* gene [25]. Tumor-bearing mice received i.p. injections of manumycin daily, using 1–5 mg/kg. Growth of inoculated tumors was inhibited significantly by manumycin treatment, and it did not have apparent *in vivo* hepatotoxicity.

Manumycin interferes with FTase activity by competing with FPP, but not the farnesyl acceptor tetrapeptide on Ras, as an enzymatic substrate for FTase [57, 68]. Other fungal metabolites that inhibit FTase by mimicking and competing with FPP at the active site of the enzyme include oreganic acid, a sulfated tricarboxylic acid [69], and trichostatin A, which induced reversion of *ras*-transformed NIH 3T3 cells to a normal morphology [70]. Barceloneic acid is a diphenyl ester, isolated from *Phoma* genus fungal extracts, that is an inhibitor of FTase [71].

Other biological metabolites with less clearly defined mechanisms of FTase inhibition also exist. These include andrastatin D from the *Penicillium* species FO-3929 [72] and RPR113228 from *Chrysosporium lobatum* [73].

SYNTHETIC INHIBITORS OF FTase ACTIVITY

Organochemically synthesized inhibitors of FTase catalytic function have been isolated. For example, valinoctin A and its analogues [74] and chaetomellic acid A anhydride [75] are FTase inhibitors.

PRENYL-BASED TRANSFERASE INHIBITORS

Several research groups have devoted their efforts to the rational design and synthesis of FPP-based inhibitors of FTase and subsequently analyzed the biological activities. The first of these to be described was termed "compound 3," and was comprised of a β-carboxylic phosphonic acid-type pyrophosphate (PP) surrogate connected by an amide linker to the hydrophobic farnesyl group [76]. Compound 3 is a potent (IC_{50} = 75 nM) and selective *in vitro* inhibitor of FTase, and the PP surrogate group is essential to its activity. Other reports [77, 78] show α-hydroxyfarnesyl phosphonic acid to be an effective inhibitor of FTase. Using a commercial STase enzyme assay kit, a recent study [79] demonstrated that using β-ketophosphonic acid resulted in a marked increase of inhibitory activity over β-hydroxyphosphonic acid and that incorporation of fluorine in the α-position led to increased inhibitory activity compared with nonfluorinated analogs. FPP mimics such as these may become useful as small, nonpeptide, anti-tumor agents.

Scanning of a synthetic tetrapeptide combinatorial library for inhibitors of FTase resulted in sixteen different consensus sequence-derived tetrapeptide analogs being synthesized and tested [77]. All were active and showed *in vitro* IC_{50} values ranging from low nanomolar to low micromolar concentrations. The most inhibitory peptide analog was D-Trp-Met-4-chlorophenylAla-L-γ-carboxyglutamic acid. It had a K_i of 2 nM and was very selective for FTase, showing little inhibitory activity against GGTase (IC_{50} > 50 μM). In contrast to CAAX-based peptidomimetics, semipeptides, and pseudopeptides (see below), this peptide analog seemed to mimic the isoprenoid substrate FPP because it demonstrated competitive inhibition of FPP and not CAAX with respect to FTase [77].

Using a technique developed to study the efficacy of different competitors, photoreactive analogs of prenyl-diphosphates were tested for their abilities to inhibit the enzymatic activity of prenyltransferases [78]. A putative GGPP analog, diazo-trifluoropropionyloxy-farnesyl diphosphate (DATFP-FPP), was developed that effectively inhibited both FTase and GGTase I, with K_i values of 100 and 18 nM, respectively. DATFP-FPP is also an effective photoreactive probe of the prenyl diphosphate binding domains of FTase and GGTase I.

CAAX-BASED INHIBITORS OF TRANSFERASE ACTIVITY

The other substrate in the transferase-mediated prenylation reaction, namely the CAAX tetrapeptide motif, has also been used as a model in the design of competitive inhibitors for the active sites of FTase and GGTase. Selective FTase inhibitors mimic the CAAX motif on the Ras protein to which the farnesyl group is added and compete for the catalytic binding site on FTase. These drugs may use an authentic amino acid sequence, such as CVLS or CVIM [63, 80], and serve as an alternative substrate that becomes farnesylated, or they may use a nonsubstrate sequence, such as CVFM [31], that does not undergo farnesylation. To prevent metabolic degradation *in vivo* prior to enzymatic target interaction, various strategies have been employed. These include incorporation of altered or nonphysiological amino acids at one or more of the four CAAX positions to make a semi- or pseudopeptide construction of a synthetic nonpeptide that is peptidomimetic towards the FTase catalytic site, with the intent of evading proteolytic hydrolysis. In addition, although quite a few compounds show high FTase inhibitory activity using cell lysates or purified enzyme, they do not readily permeate the plasma membrane of viable cells to gain access to the target enzyme *in vivo*. Therefore, prodrugs are formulated by introduction of a moiety that facilities membrane permeabilization.

Pepticinnamin E is an actinomycete metabolite composed of five amino acids linked to *o*-pentenylcinnamic acid that contains a pentapeptide sequence enabling it to inhibit FTase activity [81].

SEMIPEPTIDE AND PSEUDOPEPTIDE CAAX-BASED TRANSFERASE INHIBITORS

Semipeptide analogs of the CVFM nonsubstrate FTase inhibitor were prepared [82, 83]. Substitution of tetrahydroisoquinoline-3-carboxylate (Tic) for phenylalanine (F) yielded the CysValTicMet semipeptide that was a potent FTase inhibitor *in vitro*. However, degradation by aminopeptidases and weak cell internalization made this compound unsuitable for *in vivo* application. A further modification was made such that valine-methyleneamine isostere was substituted for valine (Val). This derivative inhibited FTase *in vitro* with an IC_{50} of 0.6 nM and inhibited anchorage-independent growth of *ras*-transformed NIH 3T3 cells with an IC_{50} of 5 μM. Replacing the A1 side chain with a *tert*-butyl group and replacing X with glutamine gave a growth IC_{50} of 190 nM, a 26-fold improvement. A prodrug was formulated by conversion of methionine (Met) into a methyl ester, and it was evaluated for *in vivo* anti-tumor activity in an athymic mouse xenograft model using human H-*ras*-transformed cells. When given twice per day at 45 mg/kg for 11 days, the semipeptide FTase inhibitor significantly extended survival.

HR-11 is another semipeptide derived from CVFM [84]. It blocked Ras farnesylation *in vitro* with an IC_{50} of 1.2 nM. The cell-permeable methyl ester HR-12 derivative had a 10 μM IC_{50} in intact cells. HR-12 had no detectable activity toward geranylgeranylation of the Ras-counteracting Rap1 protein.

It appears that improved bioefficacy of FTase inhibitors can be achieved by analogs that use: (1) aminoterminal modifications; (2) pseudopeptides or non-natural amino acids to decrease proteolytic susceptibility; (3) introduction of hydrophobic aliphatic chains to increase internalization and metabolic stability; (4) deletion of the carbonyl group between the first two residues of the tetrapeptide [85]; and (5) transformation into prodrugs using structural modification to introduce a methyl ester [82, 84] or homoserine lactone [85] group.

Pentapeptide derivatives lacking cysteine were developed as potent FTase inhibitors *in vitro* [24]. One of them demonstrated an IC_{50} of 20 nM. Its formula is Cbz-His-Tyr (OBn)-Ser-(OBn)-Trp-D-Ala-NH_2. These compounds are classified as pseudopeptides.

PEPTIDOMIMETIC CAAX-BASED INHIBITORS OF TRANSFERASE ACTIVITY

Peptidomimetics are able to substitute for the Ras C-terminal CAAX motif to inhibit its prenylation even though they are nonpeptidic synthetic organic compounds. Selective competitive inhibition of FTase results from particularly precise conformational reciprocity with the active site for farnesyl transfer catalysis. The first report of this methodology was published in 1995 [62]. A peptide mimic of the CVIM farnesylation site of Ras was designed that had the VI dipeptide replaced by aminobenzoic acid derivatives. Although this was a potent inhibitor of FTase *in vitro*, it had undesirable peptide features that hampered its use *in vivo*. The researchers then developed the first nonpeptidic mimics of CAAX by replacing the AAX tripeptide with biphenyl derivatives. The biphenyl group contained no amino acids and was linked to cysteine through a secondary amine. Thus, this compound lacks peptidic attributes and contains no hydrolyzable bonds. This peptidomimetic showed FTase inhibitory activity *in vitro* ($IC_{50} \sim 100$ nM) and also disrupted Ras farnesylation in whole cells. This CAAX mimetic demonstrated >500-fold higher potency for inhibition of FTase over GGTase I. The peptidomimetic inhibitor was not metabolically inactivated by aminopeptidases or processing by FTase and did not require a prodrug strategy for use *in vivo*. The authors concluded that peptidomimetics require free thiol and carboxylate groups separated by a hydrophobic moiety and that the conformational three-dimensional positioning of these functional groups must closely correspond to those of the authentic CAAX substrate.

The L-739,749 peptidomimetic FTase inhibitor causes rapid morphological changes and anchorage-independent growth inhibition of H-*ras*-transformed human fibroblasts in culture [86–88]. Morphological reversion to an adherent phenotype occurred within 18 hr of L-739,749 addition.

The reverted phenotype was stable for several days in the absence of inhibitor before the transformed phenotype reappeared. Cell flattening was accompanied by actin stress fiber formation in *ras*-transformed and normal human Rat-1 fibroblasts. Although a single treatment with L-739,749 caused morphological reversion, repetitive treatments were needed to reduce the growth rate significantly. Thus, the effects of L-739,749 on *ras*-transformed cell morphology and cytoskeletal actin organization could be separated from the effects on cell growth. Rho proteins regulate stress fiber formation, and RhoB is farnesylated *in vivo* [30]. One report [65] demonstrated a reduction in the ability of L-739,749 to inhibit Ras farnesylation in *ras*-transformed cells having farnesyl-independent RhoB kinase. It appears that the interplay of morphological reversion and growth suppression may utilize a complex feedback control mechanism involving farnesylated Ras and Rho proteins. In cell culture, L-739,749 also suppressed the growth of a human pancreatic adenocarcinoma cell line harboring mutant *ras*, *myc*, and *p53* genes [87].

The L-739,749 FTase inhibitor is able to cause tumor regression of some carcinomas in transgenic murine models [89]. L-739,749 suppressed H-*ras*-transformed human Rat-1 tumor growth in nude mice by 66% compared with 33% using doxorubicin [87]. Control tumors formed by v-*raf*- or v-*mos*-transformed Rat-1 cells were unaffected by L-739,749 treatments. The drug-treated mice showed no evidence of systemic toxicity. Similar selectivity for Ras-transformed cells through FTase inhibition was obtained in culture using the L-731,735 prodrug [58]. The peptidomimetic inhibition of FTase activity is effective in reversing the transformed phenotype of v-*ras*-Rat-1 cells but not of cells transformed with v-*raf* or v-*mos* [8].

Following daily administration, the FTase-selective L-744,832 CAAX mimic caused tumor regression in v-Ha-*ras*-MMTV mice bearing palpable mammary tumors [90]. Upon cessation of treatment, tumors reappeared, the majority of which regressed with the resumption of treatment. No systemic toxicity was found during necropsy of L-744,832-treated mice. This demonstration of FTase inhibitor-mediated tumor regression suggests that such drug treatments may be safe and effective anti-tumor agents that do not necessarily induce resistance in some cancer cell types.

Growth of greater than 70% of all human tumor cell lines was inhibited by 2–20 μM L-744,832, whereas the anchorage-dependent growth of nontransformed cells was less sensitive [8]. Cells with wild-type Ras and constitutively-active PTK, in which the transformed phenotype may depend upon upstream activation of the Ras pathway, were especially sensitive to L-744,832. In all cell lines tested, FTase activity was strongly inhibited within 1 hr of treatment with the drug. The general patterns of farnesylation inhibition and specific inhibition of lamin B processing were the same in sensitive and resistant cells. Functional activity of the Ras proteins was suppressed to the same degree in sensitive and resistant cells. Nonetheless, the FTase inhibitor blocked activation of the MAPK pathway in sensitive but not in resistant cell lines. This strongly suggests that Ras isoforms that were not examined and which do not depend on farnesylation mediate oncogenic growth in a fair percentage of human tumor cell lines and normal growth of non-transformed cells. Of course, mutations downstream from Ras might remain unaffected by inhibition of farnesylation and contribute to uncontrolled cell growth.

B956 and its methyl ester prodrug B1086 are CAAX analog peptidomimetic FTase inhibitors [61, 91]. They induced flat reversion and inhibited anchorage-independent growth of *ras*-transformed and *ras*-mutant human tumor cell lines expressing different *ras* oncogenes. Growth inhibition was observed at concentrations ranging from 0.2 to 60 μM. Higher concentrations (10–80 μM) of drug were required to inhibit colony formation by human tumor cell lines without *ras* mutations. The methyl ester 1086 prodrug inhibited growth of Ras-dependent human carcinoma and sarcoma cell line tumors in nude mouse xenograft models.

The biological activities of CAAX peptidomimetics can be improved by replacement of the A1A2 dipeptide with AMBA (3- or 4-aminomethyl benzoic acid) or ABA (3- or 4-aminobenzoic acid) [80]. Cys-4-ABA-Met (IC_{50} = 50 nM) showed a 128-fold greater potency as an FTase inhibitor than Cys-3-ABA-Met even though they differed only in the substitution pattern around the phenyl ring. This demonstrated that peptidomimetics require precise structural and conformational characteristics for correct positioning of Cys and Met.

Replacement of the central dipeptide with the hydrophobic amino-carboxymethyl-dihydrophenyl-benzodiazepinone yields a peptidomimetic inhibitor, Cys (BZA) Met, of moderate potency toward FTase [92, 93]. *N*-Methylation of the Cys amide of Cys (BZA) Met improved its potency by 100-fold, resulting in an *in vitro* IC_{50} of less than 1 nM. The increased affinity presumably correlates with a preferred conformation whereby it maximizes hydrophobic interaction between the BZA scaffold and the enzyme. Another report [94] showed CAAX peptidomimetics having Cys linked through a reduced pseudopeptide bond to an amino-carboxybiphenyl group to be potent FTase inhibitors that are highly selective and have little activity toward GGTase I.

FTase inhibitors have been reported to inhibit the growth of oncogenic *ras*-transformed cells at concentrations that do not affect the growth and viability of normal cells. This is despite blocking the farnesylation and membrane association of Ras in both cell types. This is a paradox given the requirement for Ras function in normal cell growth.

The BZA-5B drug is a CAAX FTase inhibitor that blocks farnesylation of H-Ras and reverses the transformed morphology of cells expressing oncogenic H-Ras [95]. BZA-5B blocks farnesylation of the lamin proteins with an IC_{50} comparable to that for H-Ras [96]. Despite potent and general inhibition of protein farnesylation, BZA-5B does not interfere with a variety of cellular functions in *ras*-

nontransformed cells. These include functions expected to be prenylation dependent, such as cell growth and viability, assembly of nuclear lamina, and membrane-association of Ras proteins. Maintenance of these events in the presence of BZA-5B is in marked contrast to their inhibition leading to morphological reversion and suppression of mitogenesis in some oncogenic *ras*-transformed cells. Non-transformed human Rat-1 fibroblasts are not affected by BZA-5B at concentrations that block growth of H-*ras*-transformed Rat-1 cells, suggesting that they possess a form of Ras whose prenylation is not blocked and has functional capabilities that overlap those of farnesylated H-Ras. A likely candidate is K-RasB, which differs from H-Ras mainly in the terminal 24 amino acids [95].

A study [95] was done on the effect of BZA-5B on prenylation of a chimeric oncogenic Ras protein designated H/K-RasBV12. This chimera consisted of the first 164 amino acids of H-Ras V12 and the last 24 amino acids of K-RasB. BZA-5B failed to block prenylation of the chimera and thus was unable to reverse the transformed morphology of Rat-1 cells expressing it. Another potent inhibitor of farnesylation, L-739,749, also failed to block prenylation of H/K-RasBV12. Thus, neither BZA-5B nor L-739,749 reversed the transformed morphology of cells expressing H/K-RasBV12. Similar results were obtained using cells transformed by a version of K-RasBV12. Resistance of K-RasB to FTase inhibitors is likely to account for resistance of non-transformed and some *ras*-transformed cells to treatment with FTase inhibitors. It is probable that isoprenylation of FTase inhibitor-resistant versions of Ras, such as K-RasB, is accomplished through geranylgeranylation by GGTase I.

Although H-*ras* oncogene mutations are the most common type in rodents, they are relatively uncommon in humans [89]. Rather, the K-Ras oncogenic product is the most relevant target in human cancers. It seems that much of the preclinical evaluation of prenylation inhibitors has been misdirected because it has been focused on inhibition of H-Ras function through blockage of farnesylation by FTase activity.

A derivative of CVIL was made that is a potent and selective inhibitor of GGTase I and that has an *in vitro* IC_{50} of 5 nM [85]. The FTase inhibitor FTI-276 is a tetrapeptide mimetic of the C-terminus of K-Ras [97]. FTI-276 prevented growth in nude mice of a human lung carcinoma that expressed the two most common human cancer genetic alterations, i.e. K-*ras* mutation and deletion of the *p53* gene. In contrast, FTI-276 did not inhibit tumor growth of a human lung carcinoma having no *ras* mutations. The CAAX peptidomimetic GGTI-287 is 10-fold more potent toward inhibition of GGTase I (IC_{50} = 5 nM) than the FTase inhibitor FTI-276 [60]. The methyl ester of GGTI-287 was 25-fold more potent in viable cells (IC_{50} = 2 μM) than the methyl ester of FTI-276 in inhibiting geranylgeranylation. Processing of H-Ras was very sensitive to inhibition by the FTI-276 methyl ester (IC_{50} = 100 nM), whereas prenylation of K-Ras was resistant (IC_{50} = 10 μM). In contrast, processing of K-Ras was much more sensitive to the GGTI-287 methyl ester (IC_{50} = 1 μM) but inhibited more weakly by the FTI-276 methyl ester (IC_{50} = 30 μM). Oncogenic K-Ras stimulation of MAPK activity was inhibited by the GGTI-287 methyl ester at concentrations (1–3 μM) that did not inhibit oncogenic H-Ras activation of MAPK [60]. This was the first demonstration of selective disruption of oncogenic K-Ras processing and signalling in viable cells using a CAAX peptidomimetic. Moreover, this report suggests that prenylation-dependent activity of K-Ras is more reliant on geranylgeranylation by GGTase I than farnesylation by FTase.

The precise mechanisms by which the GGTase inhibitor GGTI-298 and the FTase inhibitor FTI-277 (the FTI-276 methyl ester) [2] suppress human tumor growth are unclear. In human lung adenocarcinoma cells, GGTI-298 induced a G_0/G_1 cell-cycle block, whereas FTI-277 caused a G_2 block. Compactin is another GGTase inhibitor. Although FTI-277, GGTI-298, and compactin inhibited cell growth, only the GGTase inhibitors induced apoptosis. The involvement of geranylgeranylated proteins in the apoptotic pathway was confirmed by geranylgeraniol (see below) blocking compactin-induced apoptosis. It appears that protein geranylgeranylation by GGTase I is an important preventative of apoptosis. In addition, farnesylated and geranylgeranylated proteins apparently contribute to G_2/M and G_0/G_1 phase transitions, respectively.

A fluorescent assay was developed for measuring the activity of GGTase I [98]. This assay uses recombinant Ras protein with a CVLL terminal tetrapeptide (Dansyl-GCVLL) and geranylgeranyl disphosphate (GGPP) as substrates. CVFL is used as a competitive inhibitor of GGTase I in this assay, having a K_i of 200 nM. Dansyl-GCVLS, a substrate for FTase, is used as a negative control since it is inactive for prenylation under these conditions.

BISUBSTRATE INHIBITORS OF TRANSFERASE ACTIVITY

Bisubstrate analog Ras inhibitors incorporate the structural motifs of both the prenyl diphosphate and the CAAX tetrapeptide, the two substrates of the reaction catalyzed by the prenyltransferase enzyme. The simplest of these type of compounds to be used as a Ras inhibitor in a published report [99] is *N*-acetyl-*S*-*trans*-farnesyl-L-cysteine (AFC), which essentially consists of just the farnesyl molecule linked to cysteine, the amino acid to which FPP is enzymatically conjugated by FTase.

Two reports [100, 101] describe linkage of Ras CVLS tetrapeptide motifs to FPP. These bisubstrate analogs were tested using either a phosphonyl- or a phosphinyl-bearing linker, and were termed BMS-184467 and BMS-185878, respectively. The phosphonic acid analog was equipotent to the phosphinic acid analog and both exhibited higher *in vitro* selectivity for FTase versus GGTase than CVFM peptidomimetics and benzodiazepine analogs. That is, these compounds had 1000-fold more inhibitory activity toward

FTase than toward GGTase. A further 15-fold enhancement in inhibitory activity (IC_{50} = 6 nM) was achieved by replacement of the VLS tripeptide with VVM.

Methyl ester prodrugs made from these bisubstrate inhibitors inhibited FTase activity in whole cells and blocked Ras-induced cell transformation as well as clonogenicity [70, 100]. Whereas these bisubstrate analogs produced profound suppression of *ras*-transformed cell growth, they had no significant effect on untransformed NIH 3T3 cells. Inhibition of *ras*-transformed cell growth was accompanied by morphological changes, such as flattening and becoming less retractile. The cells also formed a contact-inhibited monolayer. The actin cytoskeleton reverted to an organized network of stress fibers.

The BMS-186511 methyl ester derivative of BMS-185878 affected both H-*ras*- and K-*ras*-transformed cells; however, the K-*ras* cells were less sensitive [100]. Major effects of this drug were limited to cells that principally utilize farnesylated Ras for oncogenesis, but effects were minimal in transformed cells using geranylgeranyl-Ras or myristoyl-Ras, or in cells not transformed by a Ras-dependent mechanism. These findings further support the importance of Ras types other than H-Ras and geranylgeranylation by GGTase in malignant human cells with constitutively active Ras isoforms, in addition to their involvement in normal cell division.

FTase inhibitors have been shown by many studies to block farnesylation of oncogenic Ras and suppress growth of H-*ras*-transformed cells; however, the BMS-186511 FTase inhibitor did not affect the function of the oncogenic R-Ras2 protein [102]. The apparent molecular basis for this observation is that R-Ras2 serves as a good substrate for FTase, as well as GGTase I. R-Ras2 has ubiquitous expression, regardless of whether or not it is oncogenic, and may provide an alternative to K-Ras for the insensitivity of normal and some malignant cells to FTase inhibitors.

MEVALONATE PATHWAY INHIBITION

D-Limonene and related monoterpenes, such as perillyl alcohols and γ-tocotrienol, are isoprenoids that are common constituents of fruits, vegetables, and cereal grains [60]. When used as isolated agents, these monoterpenes suppress Ras-dependent tumor cell growth both *in vitro* and *in vivo*; however, their mode of action is subject to some uncertainty as a result of contradictory reports.

Monoterpenes were shown to inhibit the growth of human pancreatic carcinoma cells with a K-*ras* mutation and an H-*ras*-transformed rat fibroblast cell line *in vitro* [103]. Limonene showed an IC_{50} for tumor cell growth of 5 mM, and perillyl and 7-methyl-perillyl alcohols, limonene oxide, and perillic acid methyl esters had IC_{50} values of 1 mM.

Limonene and perillyl alcohol showed *in vivo* efficacy as chemopreventive agents against rodent mammary carcinoma and were effective therapeutic agents toward advanced rodent mammary cancer [103, 104]. Limonene entered clinical trials in 1995 and was given orally to cancer patients; it demonstrated antitumor effects [104, 105].

Limonene and perillyl alcohol and their major *in vivo* metabolite, perillic acid, are weak inhibitors of FTase and GGTase [104]. In contrast, a minor metabolite of both, perillic acid methyl ester, is a potent inhibitor of both of these prenyltransferases. The authors claim that perillic acid methyl ester functions as a prenyl diphosphate competitive inhibitor and that commonly encountered monoterpenes represent prodrugs that are converted into pharmacologically active substances by metabolic modification. Another study [60] portrayed monoterpenes as competitive inhibitors of 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase activity. This would deplete cells of intermediate products of the mevalonate pathway and be rate-limiting both for the synthesis of cholesterol and for the production of prenyl precursors for farnesyl and geranylgeranyl diphosphates, among others [53, 59, 60, 106–108]. A third report [103] stated that, whereas the BZA-5B FTase inhibitor induced morphologic reversion of H-*ras*-transformed cells, limonene and perillyl alcohol did not. The monoterpenes also did not decrease the MAPK enzymatic activity that is downstream of Ras. Thus, although effective in inhibiting growth of tumor cells with activated Ras oncogene products, limonene and other monoterpenes are unlikely to act by inhibition of Ras function.

Lovastatin, simvastatin, pravastatin, mevinolin, and geraniol are all competitive inhibitors of HMG-CoA reductase [59, 60, 106, 108–111]. As such, they inhibit the rate-limiting enzyme for cholesterol synthesis and the mevalonate pathway. Mevalonate is the precursor of a number of substances important for the growth of cells. Among these, mevalonate synthesis is the rate-limiting substrate for the synthesis of prenylphosphates. By this means, the lovastatin-type inhibitors suppress both protein farnesylation and geranylgeranylation by decreasing cellular pools of FPP and GGPP, respectively [107]. Thus, the HMG-CoA reductase inhibitors prevent membrane-association of Ras proteins by preventing their prenylation, and this causes Ras proteins to remain nascent and nonfunctional in the cytosol [53, 106, 109, 110]. Although inhibitors like lovastatin can prevent growth of cells with oncogenic Ras proteins for obvious reasons, they can also inhibit growth of cancer cells that have no *ras* mutations, showing that suppression of cancer cell growth by these compounds is not directly dependent on the presence of a *ras* mutation [110]. This is thought to be due to suppression of the mevalonate pathway causing a broad depletion of prenyl proteins needed for a number of facets of cell growth, including functionality of various types of Ras proteins and nuclear lamins, and also cholesterol.

As a consequence of a fundamental interference in the pathways contributing to mitosis, competitive inhibitors of mevalonate synthesis arrest cells at the G_1/S cell-cycle interface and initiate apoptosis [59]. Geraniol is an acyclic monoterpenoid alcohol that has been shown to inhibit

HMG-CoA reductase activity and arrest the growth of cultured tumor cells [59]. When dietary geraniol was fed to rodents, it suppressed the growth of hepatoma and melanoma tumors in separate groups [59].

Nonetheless, the use of lovastatin in humans as a chemotherapeutic agent has been precluded by its significant nonspecific effects. One study [107] used a combination of lovastatin and the GGTase substrate precursor GGOH to potentiate blockage of H-Ras processing while concomitantly reducing lovastatin toxicity. Cotreatment with lovastatin plus GGOH enhanced the inhibition of oncogenic H-Ras and the suppression of downstream MAPK activation; however, GGOH preserved the processing of GGTase protein substrates. Provision of GGOH reduced by 15-fold the *in vitro* cytotoxic effects of lovastatin while potentiating the effects resulting from H-Ras inhibition by lovastatin. These opposing effects are seemingly due to a GGOH metabolite simultaneously being an inhibitor of FTase while also being a substrate for GGTase [107].

The effect on cell growth was studied using a combination of the pravastatin HMG-CoA reductase inhibitor and limonene against a human hepatoma-derived cell line [111]. Pravastatin at 100 μM caused 85% inhibition of cholesterol biosynthesis and a corresponding reduction in FPP. The production of farnesylated Ras was decreased markedly by addition of 1 mM limonene, and this caused a 50% reduction in DNA synthesis. The combination of pravastatin causing reduction in FPP substrate and inhibition of protein farnesylation by limonene appeared to be responsible for diminished cell growth potential due to suppressed post-translational processing of cellular proteins, including Ras.

Paclitaxel inhibits Ras protein isoprenylation, as does lovastatin [112]. Cytoplasmic PLA_2 is phosphorylated and activated downstream in the MAPK pathway and quinacrine is a PLA_2 inhibitor [112]. The combination of lovastatin and quinacrine acted synergistically *in vitro* in reducing the viability of three human prostate cancer cell lines. A nude mouse model of human prostate cancer showed paclitaxel and quinacrine to have synergistic effects in delaying tumor growth [112].

Fmev is an inhibitor of mevalonate decarboxylase [106, 109]. Fmev blocks synthesis of downstream mevalonate products including prenyl-derived lipids, thereby preventing membrane-localization of Ras proteins. Fmev prevents conversion of mevalonate to isopentenyl diphosphate and other downstream products. This prevents the synthesis of sterol and nonsterol lipids and the prenylation of proteins. As a result, prevention of *ras*-transformed cell proliferation by Fmev is possible. Lovastatin reduces membrane-associated Ras and increases the level of cytosolic Ras, but does not diminish the total amount of Ras protein [106, 109]. In contrast, treatment of *ras*-transformed cells with Fmev substantially reduced total cellular Ras levels. This indicates that the inhibition of *ras*-transformed cell proliferation resulting from treatment with Fmev that was witnessed was due at least partially to depletion of the cellular pool of Ras proteins.

OTHER Ras FUNCTION INHIBITORS

A pyrazolo-quinoline compound, methoxy-hydroxyethoxyl-ethyl-amino-methyl-pyrazolo-quinoline (SCH51344), inhibits H-*ras* transformation-induced mitogenesis and phenotypic changes [113]. SCH51344 also suppressed growth of cells transformed with v-*abl*, v-*mos*, v-*raf*, and mutant MAPK kinase oncogenes, but cells transformed with v-*fos* were resistant. Despite this, drug treatment had no effect on cytokine-induced MAPKK, MAPK, or $p90^{rsk}$ activity. Therefore, SCH51344 appears to have a novel mechanism of action either downstream or parallel to the Ras signalling pathway.

Conophylline is a *Vinca* alkaloid that serves as another Ras inhibitor by mechanisms not understood completely [114]. K-*ras*-NIH 3T3 cells are more invasive than NIH 3T3 cells in a chemotactic invasion assay. Conophylline inhibited invasion of K-*ras*-transformed cells while having no effect on non-transformed NIH 3T3 cells. The drug induced expression of E-cadherin, but not fibronectin, on the K-*ras*-transformed fibroblasts. Mouse melanoma B16/F10 is a highly metastatic cell line that expresses oncogenic H-Ras. Conophylline induced flat morphology in the melanoma cells and inhibited their invasiveness [114]. Thus, conophylline lowers invasiveness of cells expressing K-Ras or H-Ras by reversing their neoplastic phenotypes.

EFFECTS OF ONCOGENIC Ras ON RESISTANCE TO THERAPEUTIC MODALITIES

Whereas cells transformed by mutant Ras expression are very mitogenic and metastatic compared with their normal counterparts, and are commonly resistant to eradication using chemotherapeutic drugs, possibly due to up-regulation of NF-κB activity, *ras*-transformed cells are apparently not refractory to all drug treatments.

H-*ras*-transformed NIH 3T3 cells showed a 12-fold greater sensitivity to a novel topoisomerase-1 inhibitor, NB-506 (formylamino-dihydro-dihydroxy-β-D-glucopyranosyl-indolo-pyrrolo-carbazole-dione), than did parental cells [115]. These H-*ras*-transformed cells were also 3-fold more sensitive to other topoisomerase 1 (topo-1) inhibitors, such as camptothecin and irenotecan. NIH 3T3 cells transformed by the *erbB2* oncogene were neither more nor less sensitive to the topo-1 inhibitors.

The activity level of the topo-1 enzyme was determined to be 32-fold higher in the cells with oncogenic Ras than in the parent NIH 3T3 cells, but the topo-1 protein contents were the same [115]. In a cell-free system, the topo-1 activity was increased 2-fold by the addition of activated Ras protein from the transformed cells to a parental cell lysate. The topo-1 enzyme was observed to be more phosphorylated in the *ras*-transformed cells.

Presumably related to the elevated inhibitory effect on proliferation, NB-506 also decreased the amount of GTP-bound activated H-Ras in the transformed cells [115]. This suggests that the greater inhibitory effect exhibited by NB-506 on cells with constitutively activated Ras is medi-

ated by effects on the upstream signal transduction pathway.

In contrast, expression of oncogenic Ras-family proteins is implicated in enhanced resistance of cancer cells to therapy using IR, and Ras expression also affects cell responses to chemotherapeutic inducers of oxidative stress [116–118]. Human osteosarcoma subclones that differ in EJ-Ras expression levels showed a tight correlation between the amount of Ras produced and resistance to H_2O_2, IR, and doxorubicin. The differences in response could not be explained by increased activity of anti-oxidant enzymes, such as GSH, GSH-Px, GSHSTase, or superoxide dismutase.

Inhibition of Ras processing via suppression of the mevalonate pathway using lovastatin in culture resulted in markedly increased sensitivity to H_2O_2 IR, and doxorubicin [116, 118]. Lovastatin did not alter responses of control cells that did not express EJ-Ras, or in cells with activated *met* oncogene. Lovastatin prevented membrane association but not biosynthesis of Ras, so it appears that localization of Ras to the cell membrane is critical for maintenance of the oxidant-resistant phenotype.

A confirmatory report [103] relates that inhibition of H-Ras processing by treatment of a transformed embryonic rat fibroblast cell line with FTI-277, a peptidomimetic FTase-selective inhibitor, resulted in higher levels of apoptosis after radiation exposure. Radiosensitization of control cells that did not harbor *ras* mutations was not conferred by FTI-277 treatment.

These findings would be consistent with inhibition of Ras from localizing to its normal operative milieu, preventing interaction with its upstream and downstream signal transducers and short-circuiting the pathway conferring resistance to oxidative stress-induced apoptosis. One downstream substrate that contributes to cellular protection from oxidants is NF-κB, and suppression of Ras activity would secondarily down-regulate constitutive NF-κB activity. Down-modulation of NF-κB, or other Ras-responsive oxidant-protective substrates, may explain the increased sensitivity conferred by treatment with lovastatin. Pharmacological agents that affect Ras localization may enhance tumor responses to oxidant-mediated chemotherapies and to radiotherapy.

CONCLUSIONS

There are many directions from which modulation of Ras function can be approached. Eradication of cancer cells that express constitutively active oncogenic Ras protein subtypes has been problematic due to their refractory nature to commonly utilized therapeutic regimens. Modulation of the Ras function by one of the methods described in the foregoing sections, or derivatives and combinations thereof, may provide novel means by which cancer cells with oncogenic *ras* mutations could be sensitized to chemotherapy or radiation therapy regimens. Moreover, it appears that cancer cells without *ras* oncogene mutations can also be eliminated by compounds that interfere with the mevalonate pathway, which is more fundamental to mitogenesis because it allows synthesis of sterol and nonsterol lipids and without which, for example, the many different Ras-related proteins and nuclear lamins would not be prenylated and functional. This may allow for cell-specific vector-directed sabotage of the machinery that allows the uncontrolled proliferation of cancer cells. Cytokines like TGF-β1 may sensitize cancer cells to chemotherapeutic agents or ionizing radiation by down-regulation of Ras activity.

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